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The *In Vitro* Serum Protein-Binding Characteristics of *Bis*-(2-ethylhexyl) Phthalate and Its Principal Metabolite, Mono-(2-ethylhexyl) Phthalate

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The metabolism and toxicity of the ubiquitous plasticizer, *bis*-(2-ethylhexyl) phthalate (DEHP), and its principal metabolite, mono-(2-ethylhexyl) phthalate (MEHP), have been extensively investigated. In an attempt to understand their disposition in man, we studied the *in vitro* serum protein-binding characteristics of these compounds, using ultracentrifugation and agarose gel electrophoresis. The association of DEHP and lipoproteins was shown to be highly dependent upon, and proportional to, the lipid concentration of the serum. It appears that more than half of the serum DEHP is bound to proteins with density greater than 1.21 g/mL when the concentration of cholesterol is below 300 mg/dL or the cholesterol and triglyceride total concentration is less than 600 mg/dL. As the cholesterol and triglyceride concentrations increase, the percent DEHP bound to VLDL, IDL, and LDL increases. MEHP is bound principally to nonlipoprotein constituents in the serum, and this binding distribution is unaffected by lipid concentration. The percent binding of DEHP and MEHP to individual proteins was also found to be unaffected by their concentrations in serum. These data indicate that the protein-binding characteristics of these compounds, *in vitro*, is somewhat more complex than previously reported.

Introduction

The commonly used plastic polyvinylchloride (PVC), which is naturally rigid, is made flexible by the addition of plasticizing agents during its formulation process. The most common of these agents is *bis*-(2-ethylhexyl) phthalate (DEHP), which may comprise 20 to 40% of the final product. Of particular concern is its use in the manufacture of blood bank bags, other medical devices, and in more accessible items such as food wrappers, childrens' toys, and synthetic leathers. When in contact with blood and blood products, leaching of DEHP takes place, resulting in the introduction of substantial amounts into units of blood after several hours of storage (*1–3*) and into hemodialysis patients after dialysis (*4,5*). For example, Contreras and co-workers report that the accumulation of DEHP in the plasma of PVC-stored whole blood was 3.6 μg/mL/day if the anticoagulant was acid-citrate-dextrose, and 2.7 μg/mL/day in the presence of citrate-phosphate-dextrose (*6*).

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The biological effects of DEHP have been studied in numerous tissue and organ systems, as well as in a variety of species, the results of which have been thoroughly reviewed (*7,8*). The acute toxicity is very low, but chronic exposure trials in rats demonstrated progressive enlargement of the liver, induction of the cytochrome P-450 enzyme system, and proliferation of hepatic peroxisomes. Most importantly, chronic exposure for 2 years at very high doses produced hepatocarcinoma in mice and rats (*9*).

Clearly, the ubiquitous nature of this compound makes it certain that everyone is constantly exposed. An important question is, What is its concentration in man at any one time and where is it located? In an attempt to understand its disposition in man, we studied the serum protein-binding characteristics of DEHP and its principal metabolite, mono-(2-ethyhexyl) phthalate (MEHP).

Previous studies indicated that DEHP is predominantly bound to lipoprotein in the blood (*2,3,10–15*), whereas MEHP has an affinity for albumin (*15–17*). Jaeger and Rubin (*2*) have shown that in human plasma fractionated from 1 pint of whole blood stored for 14 days, 29% of the DEHP is associated with proteins with density greater than 1.21 g/mL and 71% with the lipo-

protein fraction ($d < 1.21$ g/mL). Albro and Corbett indicate that more than 80% of DEHP in plasma is associated with the lipoproteins (*15*). This study, however, used a combination of precipitation techniques and glass bead chromatography to separate the protein fractions. Dissociation of the plasticizer from the protein and redistribution are likely complications of these techniques.

Pike and co-workers (*18*) reported that DEHP displaces basic drugs from serum binding sites, ostensibly on orosomucoid. Acidic drugs were unaffected. The reported effect was minor, however. For example, the greatest effect was on the binding of quinidine at 10 μmole/L, which was reduced from 74% to 67% as the concentration of DEHP increased from 0 to 0.5 mmole/L. Further increases in DEHP concentration up to 2.5 mmole/L produced no further changes in the bound fraction of quinidine.

In related observations, Sasakawa and Mitomi reported that the rate of extraction of DEHP by blood, from PVC bags, was directly related to the lipid concentration of the blood (*10*). Pollack and co-workers reported that the rate (but not the amount) of extraction of DEHP from plasma varies inversely with the sum of the cholesterol and triglyceride concentrations (*19*).

We examined the *in vitro* serum protein binding of DEHP and MEHP using a combination of ultracentrifugation and agarose gel electrophoresis. We have also studied the effect of serum lipid concentration on the protein distribution of DEHP and MEHP. Our investigation of the binding of DEHP is relevant when considering its introduction via blood transfusion or dialysis. Serum protein binding of MEHP is pertinent when ingestion is the principal route of introduction of DEHP, given that DEHP is extensively hydrolysed in the gut (*20*).

Materials and Methods

DEHP and MEHP

Unlabeled DEHP was obtained from Aldrich Chemical Company (Milwaukee, WI). ^{14}C-DEHP was obtained from Dupont/NEN Products (Boston, MA) as the carbonyl-^{14}C form. Purity of each was confirmed by thin-layer chromatography. ^{14}C-Labeled and unlabeled MEHP were synthesized and purified as previously described (*21,22*). The identity of the synthesized product was confirmed by infrared spectrophotometry. Thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC) were used to demonstrate that the product was free of phthalic acid, phthalic anhydride, and DEHP. The specific activity of ^{14}C-MEHP was 1.1 mCi/mmole.

Specimen Preparation for Ultracentrifugation and Electrophoresis

Fasting serum specimens from hospital in-patients on which cholesterol and triglyceride assays had been ordered and performed were pooled to achieve samples with various concentrations of cholesterol and triglyceride. Cholesterol analysis was performed using a cholesterol ester hydrolase/cholesterol oxidase method on the American Monitor KDA (American Monitor, Indianapolis, IN). Triglyceride was determined with an enzymatic method using Sigma reagents (Sigma Diagnostics, St. Louis, MO).

The original ampule of ^{14}C-DEHP (0.49 mCi, specific activity = 32.6 mCi/mmole) was diluted to 10 mL with acetone (stock solution), and a working solution was prepared as needed (100 μL of stock into 10 mL of acetone). The final concentration of DEHP in the working solution was 5.9 ng/μL. Then, 75 μL (0.4 μg DEHP) of the working solution was added to 5-mL serum aliquots and incubated for 16 hr at 37°C under an atmosphere of 5% CO_2 to maintain constant pH. To study the effect of DEHP concentration, labeled and unlabeled DEHP were added to the incubation mixture to yield the following concentrations: 14.8 ng/mL, 0.99 μg/mL, 24.5 μg/mL, 49.0 μg/mL, 0.49 mg/mL, and 0.98 mg/mL. At the end of the incubation period, the specimens were subjected to density gradient ultracentrifugation and agarose gel electrophoresis. Specimen stability at refrigerator temperatures was tested by labeling serum samples as described above, and then fractionating after 0, 24, and 48 hr of storage at 4 to 6°C.

Similarly, 25-μL aliquots of purified ^{14}C-MEHP were added to 5 mL serum aliquots and were incubated as described above. The concentration study was accomplished by spiking sera with labeled and unlabeled MEHP to achieve specimens with the following concentrations: 25, 30, 75, 225, 525, and 1025 μg/mL.

Ultracentrifugation and Electrophoresis

Density gradient ultracentrifugation was performed following the protocol of Terpstra et al. (*23*). After centrifugation, the fractions were isolated and counted by liquid scintillation to determine the position of the radioactively labeled moieties. The following densities were used to define the various centrifugal zones: VLDL = $d < 1.00$ g/mL; IDL = 1.006–1.019 g/mL; LDL = 1.019–1.063 g/mL; HDL_2 = 1.063–1.125 g/mL; HDL_3, = 1.125–1.21 g/mL; plasma proteins > 1.21 g/mL. As a control for the ultracentrifugation procedure, specimens spiked with ^{3}H-cholesterol and ^{14}C-phenytoin were incubated and fractionated. Distribution was then compared to the previously reported data for these compounds.

Agarose gel electrophoresis was accomplished using established techniques, with the Corning Electrophoretic system (Corning Medical and Scientific, Medfield, MA). Controls, sera incubated with ^{14}C-DEHP as above, as well as unincubated (cold) sera, were all run in duplicate on each membrane. After electrophoresis, the control and cold sera zones were stained with Fat Red 7B for visualization of lipoproteins and with Amido Black 10B for the staining of total serum proteins. The channels containing the labeled specimens were cut into 5-mm strips. They were placed in liquid scintillation

Table 1. Protein binding of DEHP demonstrated by density gradient ultracentrifugation.

Spec ID	Cholestrol, mg/dL	Triglyceride, mg/dL	% VLDL IDL + LDL	% HDL_2	% HDL_3	% Serum protein
PD#5	156	105	18.5	6.0	3.3	72.0
PE#4	176	116	21.2	5.1	4.5	69.3
PC#4	180	125	25.2	6.6	4.0	64.2
PF#5	198	106	18.1	7.1	3.5	71.3
A#1	213	88	25.3	5.3	5.4	64.0
PJ#4-3	228	149	25.9	7.2	3.3	63.4
PB#2	275	220	27.1	4.6	3.0	65.2
PA#6	295	273	32.2	6.7	3.9	57.2
#2	310	295	51.7	2.2	2.9	38.5
#1	330	305	68.1	4.0	3.0	25.0
PG#6-3	342	508	46.7	4.6	3.5	45.1
IST#1	376	553	68.3	2.7	2.8	23.0

cocktail and counted for 10 min or 10,000 counts. Electrophoretic migration for a given labeled component was then compared to the staining pattern of the cold serum specimen to which it corresponded.

Results

The control experiment for the ultracentrifugation procedure showed ^{3}H-cholesterol binding predominantly to the VLDL, IDL, and LDL fractions (84.4%), with some binding to HDL (13.6%), and the remainder in the nonlipoprotein fraction (2.0%). The ^{14}C-phenytoin was found principally in the plasma protein fraction (96.0%), with the remaining 4.0% distributed among the lipoprotein fractions.

Table 1 shows the data for 13 human serum specimens that were incubated overnight with ^{14}C-DEHP then subjected to density gradient ultracentrifugation. It appears from these data that more than half of the serum DEHP binds to proteins with $d > 1.21$ g/mL when the concentration of cholesterol is below 300 mg/dL. Our results show that as cholesterol concentration increases, the percent DEHP bound to the VLDL, IDL, and LDL

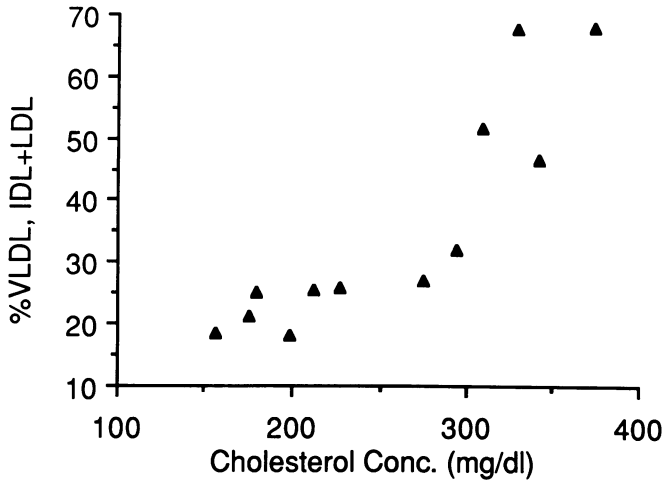

FIGURE 1. Cholesterol concentration versus percent DEHP bound to VLDL, IDL plus LDL.

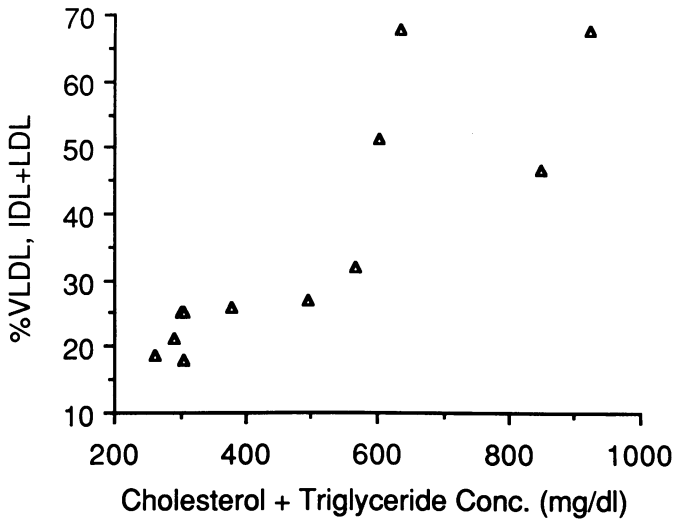

FIGURE 2. Total concentration of cholesterol plus triglyceride versus percent DEHP bound to VLDL, IDL plus LDL.

increases concomitantly ($r = 0.88$) (Fig. 1). We obtained similar results when comparing triglyceride concentration and the binding of DEHP to these fractions ($r = 0.82$). It should be noted, however, that triglyceride and cholesterol are not independent variables for our sample set. Using the total cholesterol and triglyceride concentration versus percent DEHP bound to these constituents does not significantly alter the correlation coefficient ($r = 0.86$) (Fig. 2).

Our studies also showed that when serum specimens were left at 4 to 6°C for $t = 24$ and $t = 48$ hr, the protein binding pattern of DEHP did not change from that at $t = 0$ hr, indicating stability of the DEHP-protein complex at these temperatures.

Four of the thirteen specimens were also electrophoresed in agarose gel (Fig. 3). Table 2 lists the counts per minute recovered from the 5-mm strips cut from the radioactive channel of the electrophoresis membrane. The numbers are the average of duplicate experiments. Also listed is the percentage of total recovered counts corresponding to each strip. The location of major pro-

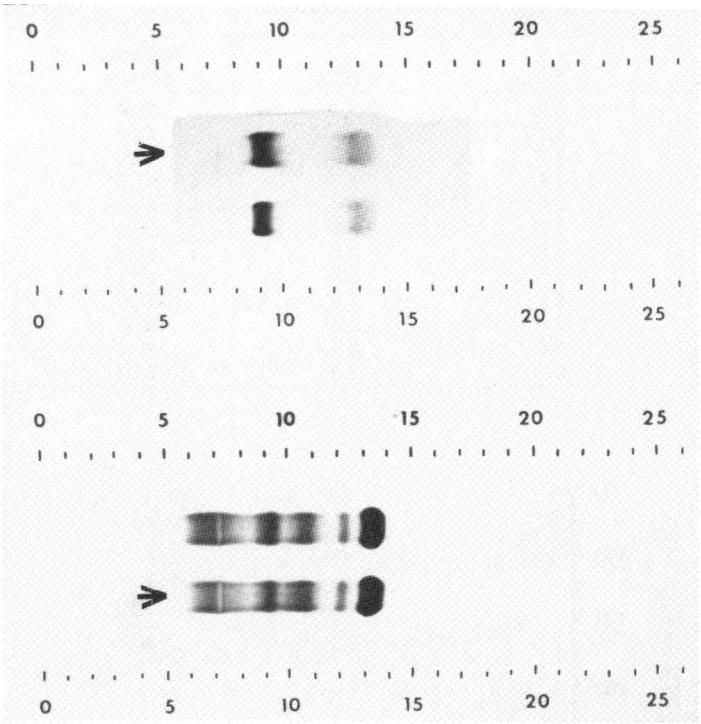

FIGURE 3. Agarose gel electrophoresis of sample serum before incubation with ^{14}DEHP and control serum (indicated by arrows), stained for lipoproteins (above) and total serum proteins (below). Ruled lines indicate fractions taken for liquid scintillation counting.

tein and lipoprotein factors with respect to the 5-mm strips is indicated in the table.

The results from these experiments parallel the ultracentrifugation data showing the greater amount of radioactivity in the β, α_2, and prealbumin regions. The actual percent radioactivity in the α_2 (pre-beta) region (mean = 38%, n = 8) indicates somewhat more binding than is evidenced by the centrifugation data for VLDL, IDL, and LDL, suggesting that there may be some binding of DEHP to nonlipoprotein constituents that migrate in the α_2 region. A major portion of nonlipoprotein bound DEHP appears to be bound to prealbumin.

Ultracentrifugation data for serum incubated with ^{14}C-MEHP (Table 3) shows preferential binding to plasma proteins (d < 1.21 g/mL), regardless of the cholesterol and triglyceride concentrations. Average plasma protein binding for all ^{14}C-MEHP specimens = 73.9 ± 2.7% (n = 8). Of the remaining 26.1% of ^{14}C-MEHP that is bound to lipoproteins, 15.3% is bound to HDL_3.

Increasing the concentration of DEHP or MEHP in the serum samples did not affect the protein-binding pattern of these compounds. For a specimen with cholesterol of 376 mg/dL and triglyceride of 553 mg/dL, the average percent DEHP bound to VLDL, IDL, and LDL = 68.3 ± 5.0% (n = 6) as the DEHP concentration was increased from 14.8 ng/mL to 0.98 mg/mL. Similarly, for a specimen with cholesterol of 211 mg/dL and triglyceride of 134 mg/dL, MEHP showed an average plasma protein binding of 84.5 ± 2.6% (n = 6) as the MEHP concentration was increased from 25 to 1025 μg/mL.

Discussion

The results of this study indicate that the distribution of DEHP among human serum proteins *in vitro* is somewhat more complex than previously reported. The observation that DEHP preferentially associates with proteins of d > 1.21 g/mL when cholesterol concentrations were less than 300 mg/dL contrasts with the previous reports (*vida supra*) suggesting that DEHP, in blood, is primarily bound to lipoproteins. These earlier studies, however, did not indicate the total cholesterol and triglyceride content of the tested specimens. The results of our ultracentrifugation studies indicate that the fraction of DEHP that binds to VLDL, IDL, and LDL is

Table 2. The distribution of ^{14}C-DEHP on agarose electrophoresis with respect to the major protein and lipoprotein fractions.

Lipoprotein regions	Zone	PA, CPM[a]	PA, %[b]	PB, CPM	PB, %	PC CPM	PC, %	PE, CPM	PE, %	Serum protein regions
	6.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	γ
	7.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	γ
β	8.0	132.00	1.10	138.00	2.02	585.00	4.60	329.00	5.28	β
β	9.0	891.50	7.46	266.50	3.90	643.00	5.06	366.00	5.87	β
β	10.00	2838.50	23.74	1364.50	19.98	3316.00	26.08	1506.00	24.15	α_2
	11.00	2712.00	22.69	1142.00	16.72	1143.00	8.99	624.00	10.01	α_2
α	12.00	434.00	3.63	192.00	2.81	678.00	5.33	261.00	4.19	α_1
α	13.00	488.00	4.08	536.50	7.86	1445.00	11.37	491.50	7.88	Albumin
	14.00	1976.50	16.00	2869.00	42.01	4580.00	36.03	1715.50	27.51	Prealbumin
	15.00	1609.00	13.46	268.50	3.93	282.00	2.22	731.50	11.73	Prealbumin
	16.00	719.00	6.01	50.00	0.73	16.50	0.13	187.50	3.01	
	17.00	96.50	0.79	2.00	0.03	10.50	0.08	21.50	0.34	
	18.00	23.50	0.20	0.00	0.00	9.50	0.07	2.00	0.03	
	19.00	13.00	0.11	0.00	0.00	4.00	0.03	0.00	0.00	
	20.00	11.00	0.09	0.00	0.00	0.00	0.00	0.00	0.00	
	21.00	7.50	0.06	0.00	0.00	0.00	0.00	0.00	0.00	

[a] CPM, counts per minute.
[b] %, percent of total counts.

Table 3. Protein binding of MEHP demonstrated by density gradient ultracentrifugation.

Spec ID	Cholestrol, mg/dL	Triglyceride, mg/dL	% VLDL, IDL + LDL	%HDL_2	% HDL_3	% Serum protein
PD#5	156	105	3.1	6.0	14.7	76.1
PC#3	180	125	6.7	3.7	13.8	75.9
PF#3	198	106	4.2	4.9	16.0	74.8
PB#2	275	220	6.1	5.6	15.1	73.0
PA#1	295	273	7.1	6.6	15.7	70.5
PI#3-3	333	516	4.0	3.5	14.7	77.7
PG#1-3	342	508	3.8	8.7	17.5	70.0
PH#2-3	369	315	5.4	6.5	14.7	73.4

positively related to the serum lipid concentration, and approaches 0.5 as the cholesterol concentration approaches 350 mg/dL or as the sum of cholesterol and triglyceride concentrations approaches 700 mg/dL. It is difficult to separate the effect of cholesterol from triglyceride concentrations, as these are dependent in our sample series ($r = 0.92$).

Four of the ^{14}C-DEHP spiked specimens analyzed by ultracentrifugation were also analyzed by agarose gel electrophoresis. The cholesterol concentration of these samples ranged from 176 mg/dL to 295 mg/dL, which is a narrower range than the series used for ultracentrifugation, and the correlation between lipid concentration and percent of DEHP bound to the lipoprotein fractions is not apparent from the electrophoresis data. It should be noted, however, that this correlation was also not apparent from the ultracentrifugation data on the same specimens, i.e., those specimens with cholesterol concentrations of less than 250 mg/dL (Fig. 1). The relatively weak dependence of percent DEHP binding to lipoprotein on lipid content when the sample cholesterol concentration is less than 250 mg/dL, with a much greater slope above 250 mg/dL (Fig. 1), is suggestive of at least two lipoprotein sites of DEHP affinity. Cholesterol in the low-density lipoprotein molecule has been shown by ^{13}C-NMR to be in two forms (*24*). The major portion is on the surface of the particle, with lesser amounts in the core. There is an interfacial flux of the surface cholesterol of both HDL and red blood cells (*25*); also, the highly lipophilic DEHP may be solubilized with cholesterol ester and triglyceride in the lipoprotein core.

By contrast, the fraction of MEHP bound to lipoprotein was approximately 0.25, most of which was associated with HDL, and was independent of the sample lipid concentration. The remaining three-fourths was bound to other serum proteins. That MEHP is less strongly associated with lipoprotein than DEHP is not surprising, in light of the diminished lipid solubility due to its free carboxyl group and greater polarity. Also, the free carboxyl group makes likely a binding to albumin analogous to that of fatty acids.

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